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Chromosomal Position and Specific Demethylation in Enhancer Sequences of Germ Line-Transmitted Retroviral Genomes During Mouse Development

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The methylation pattern of the germ line-transmitted Moloney leukemia proviral genome was analyzed in DNA of sperm, of day-12 and day-17 embryos, and of adult mice from six different Mov substrains. At day 12 of gestation, all 50 testable CpG sites in the individual viral genomes as well as sites in flanking host sequences were highly methylated. Some sites were unmethylated in sperm, indicating de novo methylation of unique DNA sequences during normal mouse development. At subsequent stages of development, specific CpG sites which were localized exclusively in the 5′ and 3′ enhancer regions of the long terminal repeat became progressively demethylated in all six proviruses. The extent of enhancer demethylation, however, was tissue specific and strongly affected by the chromosomal position of the respective proviral genome. This position-dependent demethylation of enhancer sequences was not accompanied by a similar change within the flanking host sequences, which remained virtually unchanged. Our results indicate that viral enhancer sequences, but not other sequences in the M-MuLV genome, may have an intrinsic ability to interact with cellular proteins, which can perturb the interaction of the methylase with DNA. Demethylation of enhancer sequences is not sufficient for gene expression but may be a necessary event which enables the enhancer to respond to developmental signals which ultimately lead to gene activation.

The only modified base of vertebrate DNA, 5-methylcytosine, is almost exclusively present in CpG dinucleotides. Its distribution along the chromosome is nonuniform; instead, complex patterns of methylated sites have been found by DNA sequencing (8, 42) and by analysis with restriction enzymes that fail to cut DNA when their recognition sequence is modified (for reviews, see references 1, 9 and 10). During DNA replication, these patterns are stably inherited, probably through the action of a maintenance methylase (49, 58). Changes of methylation pattern occur in somatic cells as developmentally programmed demethylation at specific sites or as random loss at multiple sites. De novo methylation of cellular sequences was detected only in repetitive sequences and only during early stages of development. Similarly, de novo methylation of foreign DNA occurs reproducibly only on transfer into early embryonic but not into somatic cells (for a review, see reference 24).

To evaluate the function of DNA methylation, both the mechanisms by which methylation patterns are established and the functional consequences of methylation at specific genomic sites have to be explored. In vitro methylation of selected CpG residues frequently leads to the suppression of gene activity (for reviews, see references 2 and 21). Foreign genes when introduced into the germ line of mice usually become de novo methylated (26). In many cases, expression of the inserted genes is suppressed at multiple chromosomal positions, and the function of regulatory sequences in specific cell types or states of development cannot be predicted (5, 15, 22, 32, 38, 51).

To deduce the mechanisms by which the methylation of individual CpG residues is determined at different chromosomal positions and at various stages of development, we used the collection of Mov mouse substrains (22). These mice each carry a single Moloney murine leukemia virus (M-MuLV) genome at a distinct chromosomal integration site. The proviruses, although flanked by their own regulatory sequences (for reviews, see references 53 and 54), appear to depend also on cellular control mechanisms; i.e, they are repressed in early embryogenesis and become activated at stages of development that are specific for individual mouse strains, suggesting an influence by the chromosomal environment on provirus expression. Furthermore, an inverse correlation between the level of methylation within the M-MuLV genome and its activity in a transfection assay suggests an involvement of DNA methylation in the cellular suppression mechanism (7, 18, 19, 26, 47, 52).

In this study we determined the methylation at more than 50 restriction enzyme sites in each of six endogenous M-MuLV proviruses and in flanking host sequences. All unmethylated sites in the viral DNAs that occur in sperm became de novo methylated at day 12 of development, whereas demethylation events were found at subsequent stages of embryogenesis. Enhancer sequences had a much higher probability of becoming demethylated than all other sites in the viral genome and the flanking sequences. However, the actual degree of demethylation of the enhancer sequences was dependent on the chromosomal position of the M-MuLV provirus and on the stage of differentiation and development. These data indicate that position-dependent methylation patterns may be involved in variations of gene expression as seen in transgenic mice. Furthermore, our results suggest that developmental demethylation events of enhancer sequences reflect specific DNA-protein interactions, for which evidence has been accumulating recently (39, 41).

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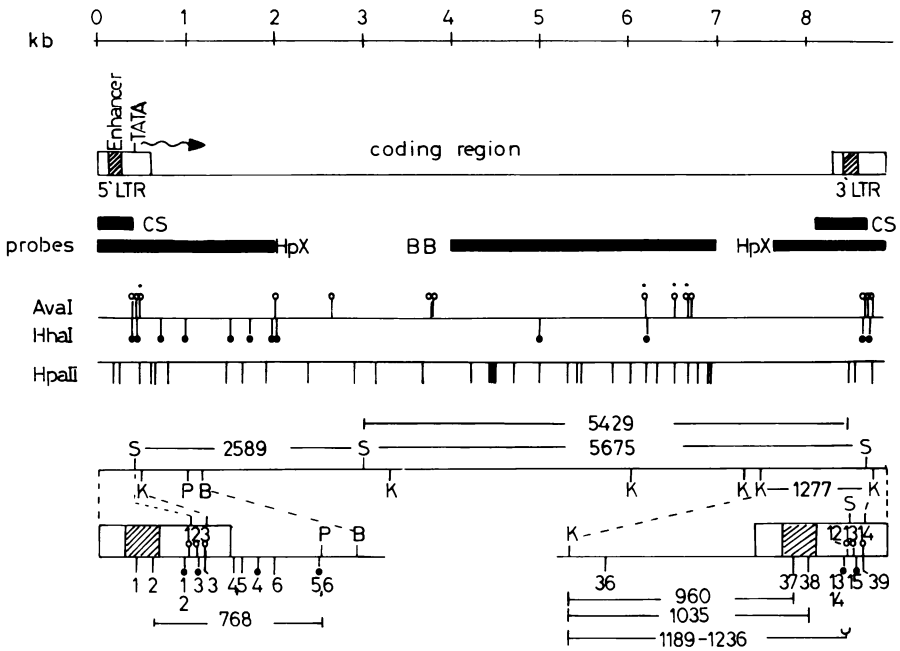

FIG. 1. Schematic representation of functional regions and restriction maps of a M-MuLV provirus. The open bar shows the LTR which carries a tandem repeat of 75 bp (striped area) with enhancer function (28). TATA marks the region 23 to 30 bp upstream of the RNA cap site; the direction of transcription is shown by the arrow. The filled bars represent the position of the *Cla*I-*Sst*I (CS), *Hpa*I-*Xho*I (HpX), and *Bam*HI (BB) fragments used as hybridization probes (see the text for details). Capital letters mark the positions of the methylation-insensitive enzymes *Sst*I (S), *Kpn*I (K), *Pst*I (P), and *Bst*EII (B). Numbers between horizontal lines indicate the length of fragments in bp; the sites of individual methylation-sensitive enzymes are numbered from the 5' end. Stars label *Ava*I sites that are also recognized by *Hpa*II. All data were taken from the published sequence of M-MuLV (46).

MATERIALS AND METHODS

Embryos and mice. The origin of the Mov substrains has been described previously (22). BALB/c and C57BL/6 mice were obtained from the Zentralinstitut für Versuchstierzucht (Hannover, Federal Republic of Germany). 129/SV ter breeding pairs were from L. Stevens. All mice were bred at the Heinrich-Pette-Institut. Embryos were obtained from timed matings, with the day of the mating plug counting as day 1 of gestation. The genotype of individual embryos derived from heterozygous parents of the Mov-13 strain was determined by analysis of DNA from individual placentas as described previously (43).

Isolation of DNA. Fetal livers, brains, and kidneys from day-12 and day-17 embryos were dissected under the microscope and immediately frozen in liquid nitrogen. Mature sperm from 2- to 3-month-old male mice were isolated from dissected vas deferens. High-molecular-weight DNA was isolated from pools of 6 to 8 day-12 embryos, from 6 to 8 brains and livers and 20 kidneys of day-17 embryos, and from pools of 4 brains or kidneys and 16 vas deferens from adult mice according to published procedures (20), with 0.14 M mercaptoethanol in the lysis buffer for sperm (27). Frozen tissues were thawed at 0°C in 10 volumes of 10 mM Tris hydrochloride (pH 8.0)–100 mM NaCl–25 mM EDTA before being subjected to Dounce homogenization in the same buffer.

Restriction enzyme analysis. The restriction enzymes *Acc*I, *Sst*I, and *Xho*I were obtained from Bethesda Research Laboratories, Inc.; *Bst*EII, *Cfo*I, *Eco*RI, *Hpa*II, and *Pvu*II were from Calbiochem-Boehringer; *Bss*HII was from New England BioLabs, Inc.; and enzymes were from either Bethesda Research or Boehringer. All digests were performed under conditions recommended by the suppliers, with 3 U of enzyme per μg of DNA and 8 to 16 h of incubation. Spermidine was added at 1 mM to all digests (3). The completeness of all reactions was controlled in samples by visual inspection of the restriction pattern derived from 0.5 μg of added plasmid DNA. DNA fragments were separated electrophoretically in 1.4% agarose gel as described previously (25), depurinated in situ in 0.05 M HCl (modified as described in reference 55), transferred to Gene Screen plus membranes (New England Nuclear Corp.), and hybridized at 65°C in Tris phosphate buffer as recommended by the supplier.

Hybridization probes. ^{32}P-labeled probes for specific regions of the M-MuLV genome were obtained by nick translation (35) of DNA restriction fragments that had been phenol purified after electrophoresis in low-melting-point agarose (Sigma Chemical Co.) gels. Probe HpX, a *Hpa*I-*Xho*I fragment of 2.65 kilobases (kb) covering the 5' and 3' ends of the M-MuLV genome, was prepared from plasmid pMo (23), that contains a circular permuted provirus. Probe BB, the 3-kb viral *Bam*HI fragment of the Mov-3 provirus, was derived from plasmid pMov-3 (18), and probe CS, the 3' viral *Cla*I-*Sst*I fragment of 0.55 kb was isolated from pTK 10/7, a plasmid carrying the *Cla*I-*Bam*HI 3' viral cellular junction fragment of pMov-9 (7). Plasmid p9-1-2, containing the 3' flanking *Bam*HI fragment of pMov-9, and the 0.56-kb *Bst*EII-*Bgl*II fragment from p13-3, which carries the *Hpa*I-*Bgl*II fragment obtained from cellular sequences around the integration sites of the Mov-13 provirus (16), were used as probes in the analysis of sequences adjacent to the M-MuLV genomes of Mov-9 and Mov-13 mice. respectively. Probe

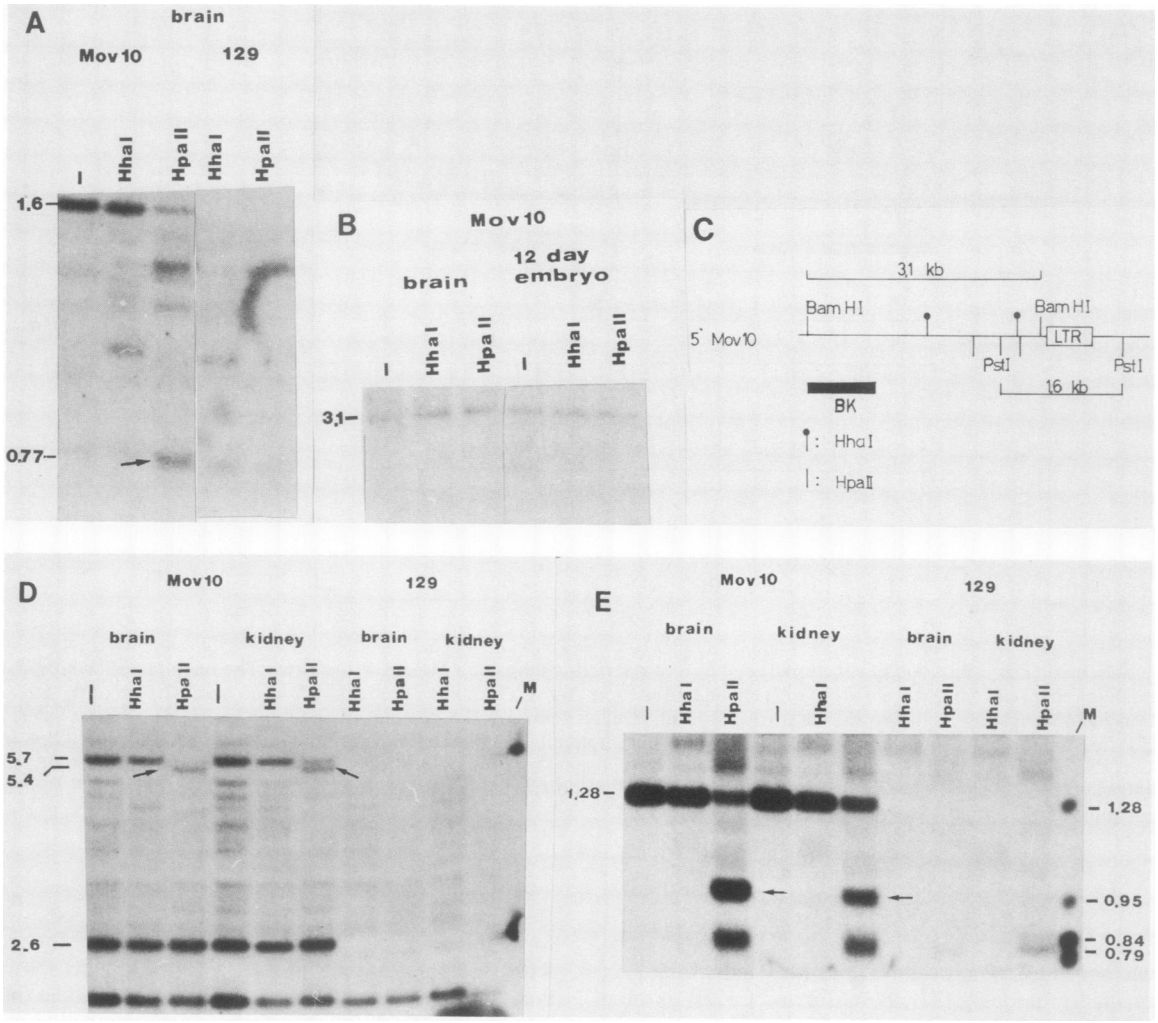

FIG. 2. Exclusive localization of unmethylated sites in the Mov-10 provirus to the enhancer regions. A 20-μg volume of DNA from Mov-10 and parental 129 mice was digested with *Pst*I (A), *Bam*HI (B), *Sst*I (D), or *Kpn*I (E) and in addition with either *Hha*I or *Hpa*II, electrophoretically separated on 1.4% agarose gels, and blot transferred to nylon membranes. M-MuLV-specific sequences shown in panels A, B, and E, were detected by hybridization with the ^{32}P-labeled HpX probe shown in Fig. 1. A 3.1-kb segment of cellular sequences 5′ to the integration site of the Mov-10 provirus was identified with probe BK (C; identical data for 129 mice not shown). The indicated lengths (in kb) of M-MuLV-sepcific fragments were determined by comparison with comigrating marker (M) fragments that were derived by digestion of the M-MuLV-carrying plasmid pMov-3 (18) with *Sst*I (D) or with either *Kpn*I (1.28 kb), *Cla*I plus AccI (0.95 kb), or *Hpa*II (0.84 plus 0.79 kb) (E). *Hin*dIII-digested lambda DNA was used in panel B (results not shown). pMov-3 (18) DNA was cleaved with either *Bst*EII (2.3, 7.7, and 0.78 kb) or *Pvu*II plus *Pst*I (1.4, 0.82, and 0.70 kb; see reference 46) for panel A (results not shown). The fragments generated by cleavage of the 5′ cellular viral *Pst*I junction fragment of 1.6 kb (17) at site *Hpa*II-2 (A) and the 3′ viral *Sst*I (D) or *Kpn*I (E) fragments of 5.7 and 1.28 kb, respectively, at site *Hpa*II-37 are indicated by arrows (compare with Fig. 1).

BK, a 0.9-kb *Bam*HI-*Kpn*I fragment specific for 5′ flanking sequences of the Mov-10 provirus, was prepared from pMov-10 (17).

RESULTS

A detailed restriction map of the M-MuLV genome is shown in Fig. 1 (46). A direct repeat of 594 base pairs (bp), termed long terminal repeat (LTR), contains all viral regulatory sequences and is present at the ends of the proviral genome. The methylation-sensitive enzymes *Hpa*II, *Hha*I, *Sma*I, and *Ava*I were used in combination with methylation-insensitive enzymes to analyze the methylation status of more than 50 CpG sites which are numbered from the 5′ end and will be referred to in the text. Probes spanning different regions of the M-MuLV proviral genome which were important in the mapping of individual restriction fragments are indicated in Fig. 1. Because these probes also cross-react with multiple endogenous murine viruses, DNA from uninfected parental mice was always used in parallel to identify M-MuLV-specific fragments.

M-MuLV genome in mice of six different Mov substrains is highly methylated at 49 sites and undermethylated exclusively in the 5′ and 3′ LTR enhancer region. The methylation status of the endogenous M-MuLV proviral genomes of six different Mov substrains was analyzed in DNA from brain and kidneys of adult mice, and results obtained with the Mov-10 strain are shown in Fig. 2. Digestion of the DNA with *Pst*I and hybridization with the HpX probe (Fig. 1) resulted in a 1.6-kb fragment (Fig. 2A) representing 1-kb viral sequences at the 5′ end of the proviral genome and 0.6-kb flanking sequences (17). The intensity of this fragment was not changed after digestion with *Hha*I, indicating methylation of

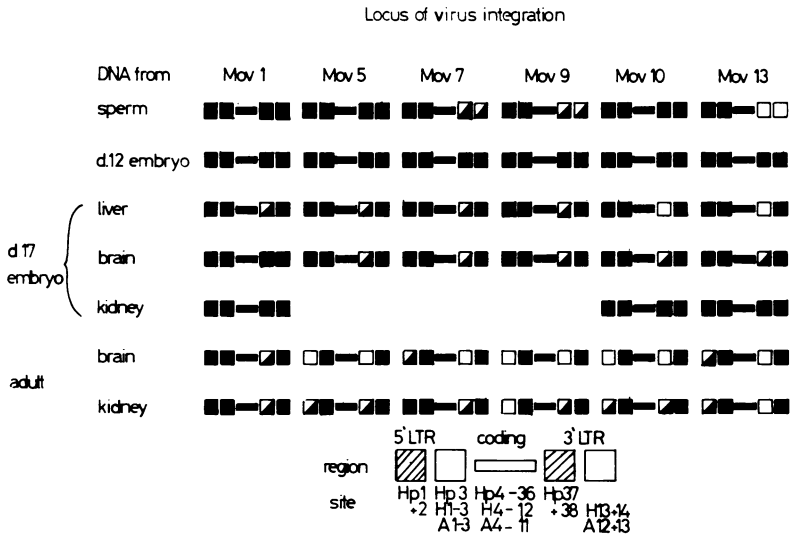

FIG. 3. Developmental changes in methylation of M-MuLV genomes located at different chromosomal positions. The methylation patterns of the single M-MuLV genome present in Mov mouse strains (22) were analyzed in sperm, day-12 embryos, and tissues of day-17 embryos and adult mice with restriction enzymes by using the hybridization probes HpX, CS, and BB shown in Fig. 1. The 5′ LTR, coding, and 3′ LTR regions were analyzed with *Pst*I, *Sst*I, and *Kpn*I, respectively (Fig. 1) and by secondary digestion with the methylation-sensitive enzymes *Hha*I (H), *Ava*I (A), and *Hpa*II (Hp) (see Fig. 1, 2, 4, 5, and 6). Sites *Hpa*II-1 to *Hpa*II-38 and *Hha*I-1 to *Hha*I-14 were analyzed in all DNAs; the methylation at *Ava*I sites 1 to 13 was determined in sperm and day-12 embryos of all strains and at all stages of development in Mov-13 mice. Filled boxes indicate 80% or more resistance to secondary digestion and demonstrate virtually complete methylation at all represented sites, including the *Hpa*II sites of the enhancer regions (striped). Half-filled boxes indicate 20 to 80% cleavage, and empty boxes indicate more than 80% cleavage within the respective M-MuLV regions due to partial methylation at any of the represented sites. In addition, site *Hha*I-15 (Fig. 1) was found methylated in adult Mov-1 and Mov-9 mice by *Eco*RI-*Hha*I analysis (data not shown). The most rightward site of the M-MuLV genome, *Hpa*II-39, which is identical to site *Ava*I-14, was analyzed in adult Mov-9 mice (Fig. 6) and in all tissues of Mov-13 mice (data not shown). It was methylated in all somatic tissues and unmethylated in Mov-13 sperm.

all six *Hha*I sites (Fig. 1), whereas digestion with *Hpa*II generated a 770-bp band (arrow) which was not detected in control DNA. This *Hpa*II cleavage site was mapped by separate analysis of the flanking and viral sequences. With probe BK (Fig. 2C, map) and *Bam*HI digestion, all *Hha*I and *Hpa*II sites within 3.1 kb of 5′ flanking sequences were found methylated (Fig. 2B), and the same result was obtained for the 2.6-kb *Sst*I fragment from the viral 5′ end (Fig. 1 and 2D). Thus, the unmethylated *Hpa*II site was localized within the first 400 bp of the LTR and corresonds to site *Hpa*II-2, which is localized 768 by 5′ of the viral *Pst*I site in the enhancer region (28, 46). The methylation of site *Hpa*II-1 could not be determined due to the small homology of the probe to the 5′ end of the 1.6-kb *Pst*I band.

All *Hha*I sites present in the 5.7-kb *Sst*I fragment representing the 3′ two-thirds of the proviral genome were methylated, while *Sst*I-*Hpa*II double digestion generated a strong cleavage product of 5.4 kb (arrows, Fig. 2D). Although this cleavage product represented only about 50% of the 5.7-kb *Sst*I fragment, no other cleavage products were seen in brain DNA. This result is most likely due to a low level of random nonmethylation in some of the 24 *Hpa*II sites present in this *Sst*I fragment and was confirmed by additional *Kpn*I digests with the BB probe (compare with Fig. 1; data not shown).

The HpX hybridization probe detects a 1.28-kb *Kpn*I fragment at the 3′ end of the proviral genome (Fig. 1) which was used to map the 5.4-kb *Hpa*II-*Sst*I fragment. Although all *Hha*I sites in this *Kpn*I fragment were methylated, *Hpa*II digestion generated a strong 950-bp cleavage product (Fig. 2E). This result indicates demethylation of site *Hpa*II-37, which is located in the enhancer region of the 3′ LTR and is 960 bp 3′ of the *Kpn*I site (compare 46). The *Kpn*I-*Hpa*II-2 fragment derived from the 5′ LTR is too small to be seen in Fig. 2E.

The analysis of the M-MuLV genomes in adult Mov-1, Mov-5, Mov-7, Mov-9, and Mov-13 mice, performed as described above for Mov-10 mice (Fig. 2), revealed a high degree of methylation in more than 50 sites on the M-MuLV proviral genome in all six Mov substrains. The only unmethlyated CpG sites were situated in the 5′ and 3′ LTR enhancer region and corresponded to sites *Hpa*II-2 and *Hpa*II-37 (Fig. 3).

Tissue-specific demethylation at the 3′ enhancer region of M-MuLV during development. The influence of cell differentiation on the M-MuLV methylation pattern was analyzed at day 17 of gestation. DNAs from liver, brain, and kidney of Mov-10 embryos were digested with *Kpn*I and hybridized to the HpX probe. Double digestion with *Hha*I showed complete methylation (Fig. 4), while *Hpa*II cleaved the 1.28-kb band almost completely in liver and partially in brain, generating subfragments of 950 and 1,050 bp that were not visible in the control DNA (arrows in Fig. 4). In contrast, Mov-10 kidney DNA was completely methylated at all *Hpa*II sites. The subfragments generated in liver and brain indicated that *Hpa*II sites 37 and 38 (compare with Fig. 1) were partially unmethlyated. The methylation in the 3′ M-MuLV enhancer region differed among liver, brain, and kidney of day-17 embryos derived from the other five Mov substrains to an extent similar to that described for Mov-10 (Fig. 3).

A comparison between fetal and adult tissues indicated a decrease in methylation with age, the brain having always a lower methylation level than the kidney (compare Fig. 4 with Fig. 2E). Similar results were obtained with the proviral

genomes in the other Mov substrains (Fig. 3), indicating a progressive and tissue-specific loss of methylation during development.

Chromosomal position of the proviral genome affects the methylation of the viral enhancers. The chromosomal position of a provirus influences the methylation pattern of the 5′ and 3′ LTR. This effect was seen by comparing individual sites of the M-MuLV genome in DNA from somatic tissues or from sperm of the different Mov stubstrains. Whereas all sites analyzed in the 3′ LTR of M-MuLV in Mov-10 sperm (Fig. 5A) were methylated, as is shown by the resistance of the 1.28-kb *Kpn*I fragment to cleavage with *Hha*I plus *Ava*I and with *Hpa*II, the corresponding *Kpn*I fragment in Mov-7 sperm DNA was more than 50% sensitive to digestion with *Hha*I plus *Ava*I and with *Hpa*II, generating specific fragments of 1.2 and 0.95 kb, respectively. The 1.2-kb fragment may be derived from sites *Hha*I-13 or *Hha*I-14 or from sites *Ava*I-12 or *Ava*I-13, as individual cleavage products would not have been resolved (Fig. 1). The 0.95-kb band, however, indicated hypomethylation of site *Hpa*II-37 (compare with Fig. 2E). Similar differences in the methylation of the 3′ enhancer regions were found in sperm DNAs from other Mov strains and were revealed in the 5′ enhancer region by analysis of somatic tissues from adult mice as well (Fig. 3). Our results therefore indicate that identical sequences in M-MuLV proviruses can have different levels of methylation in the same tissues when the sequences are integrated at different chromosomal positions on the mouse genome.

Complete methylation of all *Hha*I, *Ava*I, and *Hpa*II sites in six M-MuLV genomes of 12-day embryos indicated de novo methylation in early development. Analysis of 12-day embryos with the HpX probe revealed complete methylation of all sites within the 1.28-kb *Kpn*I fragment of Mov-7 and Mov-10 mice (Fig. 5B) and of all other Mov strains as well (Fig. 3). Thus, all sites of the M-MuLV genomes that were unmethylated in sperm (Fig. 3 and 5A) were methylated in day-12 embryos, suggesting that in early embryogenesis, all CpG dinucleotides of the M-MuLV genome become de novo methylated, irrespective of the chromosomal position of the provirus.

De novo methylation of M-MuLV genomes has been detected previously in infected embryonic carcinoma (EC) cell lines by *Sma*I digestion (12, 50). Since EC cells resemble pluripotent cells of the early mouse embryo (36), we studied de novo methylation at M-MuLV enhancer sequences in DNA from two cell clones that were derived after infection of F9 EC cells with M-MuLV and carried 10 to 50 proviral copies (50). The intensity of the 1.28-kb *Kpn*I fragment (Fig. 1) was only slightly affected by *Hpa*II digestion, indicating that *Hpa*II sites 37 and 38 were methylated in more than 80% of the proviral genomes (data not shown). Thus, EC cells have the capacity to methylate enhancer sequences of M-MuLV genomes de novo, suggesting that the genetically transmitted proviruses in Mov mice might become methylated much earlier than at day 12 of embryogenesis.

All the *Hha*I, *Ava*I, and *Hpa*II sites 5′ of the 3′ LTR were found methylated in the M-MuLV genomes of all Mov substrains analyzed in sperm and at day 12 of development (Fig. 3).

Methylation status of the M-MuLV enhancer sequences is not correlated with the methylation of flanking cellular sequences. To explore possible interactions between proviral and flanking host sequences, we determined the methylation pattern of cellular sequences in the vicinity of the LTR regions. Figure 6 shows the analysis of the 5′ and 3′ flanking regions of the Mov-9 provirus in DNAs from sperm and day-12 embryos. On digestion with *Bst*EII and hybridization with the CS probe, a 4.6-kb 3′ viral cellular junction fragment was detected (Fig. 6A). The testable sites within the 3′ flanking sequence were methylated in sperm and day-12 embryos, and no significant demethylation of these cellular sequences was found in day-17 embryos (data not shown). An analysis of the 3′ cellular sequences in DNA from adult Mov-9 brain with probe p9-1-2 (7) is shown in Fig. 6B. The 5.2-kb *Hpa*I-*Eco*RI fragment containing 1.3 kb from the 3′ end of M-MuLV was not cleaved on incubation with *Hha*I, *Sma*I, or AvaI, showing methylation at all sites in the flanking sequences (legend, Fig. 6). Since *Sma*I and *Hpa*II recognize the same single site in the 3′ flanking region (legend, Fig. 6) and also the same *Hpa*II-39 site (Fig. 1), the cleavage product C, generated by *Hpa*II, indicated nonmethylation at the *Hpa*II sites in the enhancer region of the 3′ LTR (Fig. 1), confirming the results obtained by analysis of the viral *Kpn*I fragments (Fig. 3).

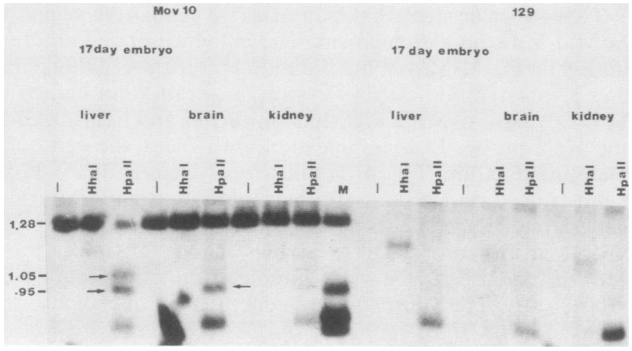

FIG. 4. Tissue-specific differences of 3′ enhancer methylation in day-17 embryos. The DNAs from tissues of Mov-10 and 129 embryos were analyzed with *Kpn*I and probe HpX (Fig. 1) as described in the legend to Fig. 2E. The amount of *Hpa*II cleavage products of 0.95 and 1.05 kb (arrows) and the resistance of the 1.28-kb 3′ viral *Kpn*I band to *Hpa*II digestion varied between liver, brain, and kidney, indicating tissue-specific levels of methylation of sites *Hpa*II-37 and *Hpa*II-38 in the 3′ enhancer region of M-MuLV (compare with Fig. 1).

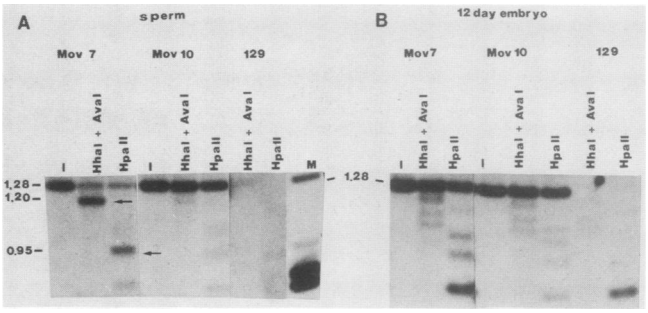

FIG. 5. Position-dependent and de novo methylation at 3′ LTR sequences. (A) Analysis of *Kpn*I-digested DNAs from sperm with the HpX probe of Fig. 1, similar to those described in the legend to Fig. 2E, revealed position-dependent methylation at *Hha*I plus *Ava*I and *Hpa*II sites in the 3′ LTR, as is shown by partial digestion of the 1.28-kb viral *Kpn*I fragment of Mov-7 but not of Mov-10 mice, yielding indicative cleavage products (arrows; compare with Fig. 1). (B) Similar analysis of DNAs from day-12 embryos showed de novo methylation at these sites, since the 1.28-kb *Kpn*I band of Mov-7 mice had become resistant to cleavage with methylation-sensitive enzymes.

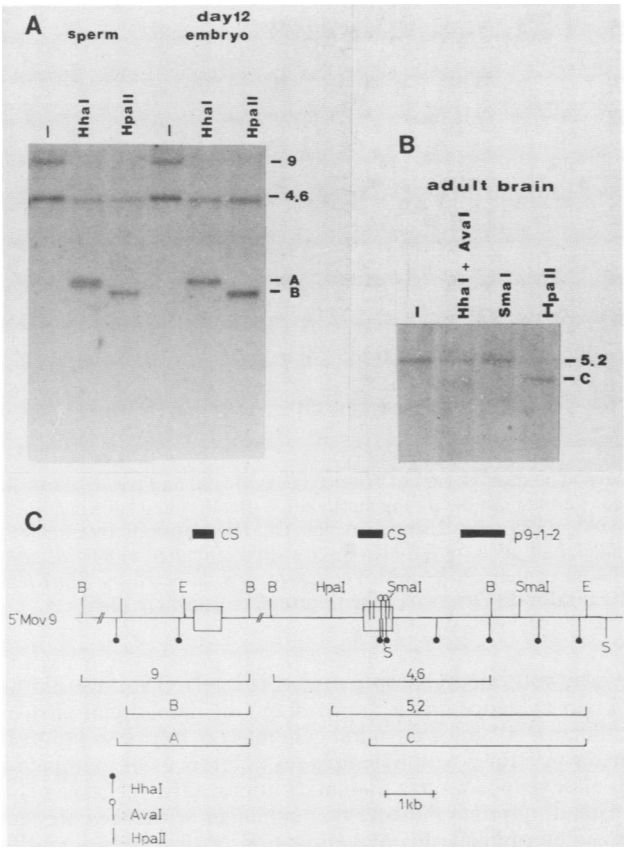

FIG. 6. Methylation at the Mov-9 locus. (A) Analysis of cellular sequences 5′ and 3′ to the M-MuLV genome of Mov-9 mice. Samples (20 μg) of DNAs from Mov-9 mice were digested with *Bst*EII (B) and with the indicated methylation-sensitive enzymes. The 5′ and 3′ viral cellular junction fragments of approximately 9 and 4.6 kb, respectively (C), were identified after electrophoresis in a 1.4% agarose gel with the CS probe shown in Fig. 1. The positions of unmethylated *Hha*I and *Hpa*II sites generating bands A and B, respectively, are shown in panel C. The 3′ flanking sequences were resistant to cleavage; the reduction in the intensity of the 4.6-kb band of sperm was due to partial cleavage in the 3′ LTR (Fig. 3), as is shown by the resistance of the 3′ *Sst*I (S) fragment by analysis with probe p9-1-2 (C; data not shown). (B) The 3′ M-MuLV LTR and 3′ flanking sequences were analyzed in 20-μg samples of DNA from Mov-9 mice by *Hpa*I-*Eco*RI (E) digestion, electrophoresis in a 1.4% agarose gel, blotting, and hybridization with ^{32}P-labeled p9-1-2, derived from the Mov-9 locus (7; compare with panel C). The viral cellular junction fragment of 5.2 kb was specifically cleaved by *Hpa*II but not by *Sma*I, generating fragment C that comigrated with the *Sst*I (S)-*Eco*RI fragment of plasmid pMov-9 that carries the *Eco*RI fragment shown in the map (7; marker not shown), indicating cleavage at the enhancer region of the 3′ LTR (open bar). *Hha*I and *Ava*I reduced the intensity of the 5.2-kb band due to random loss of methyl groups at the multiple sites for these enzymes (C).

The 5′ flanking sequences of the Mov-9 provirus present in a 9-kb *Bst*EII fragment (Fig. 6A) were completely cleaved by *Hha*I and *Hpa*II in DNA from sperm and day-12 embryos, indicating that sites at 1.4 to 1.5 kb 5′ of the 5′ LTR were unmethylated, whereas an *Hha*I site close to the LTR was methylated (legend, Fig. 6). The same result was obtained with DNAs from day-17 embryos and adult mice (Fig. 7).

All CpG sites in the 5′ flanking sequences of the Mov-10 provirus were methylated in DNA from sperm and day-12 embryos, as was shown for adult brain in Fig. 2B.

Figure 7 summarizes the methylation pattern of M-MuLV LTR and flanking cellular sequences during development and includes data from a recent analysis of 3′ flanking sequences in the Mov-13 strain (24a). Demethylation of enhancer sequences in Mov-9, Mov-10, and Mov-13 proviruses occurs independently of the methylation pattern in the respective flanking sequences, which remains unchanged.

DISCUSSION

Viral enhancer regions are specifically and exclusively demethylated in somatic tissues. The collection of Mov mouse strains, each carrying a single M-MuLV genome at a distinct chromosomal position (22), was used to deduce mechanisms involved in the establishment of methylation patterns. In six mouse strains, all 39 sites of the methylation-sensitive restriction enzymes *Hha*I, *Ava*I, and *Hpa*II that are present in the viral coding region were fully methylated at all stages of development, i.e., in sperm and in day-12 embryos, as well as in organs of day-17 embryos and of adult mice. With the exception of Mov-13 sperm, the methylation pattern of the cellular sequences adjacent to the proviral genomes, although different at individual integration sites, remained constant throughout development.

In DNA of day-12 embryos, all testable CpG sites in the coding as well as noncoding regions of the six M-MuLV proviral genomes were fully methylated. At later stages of development, demethylation of specific CpG dinucleotides occurred in the LTR sequences of all M-MuLV proviruses. In somatic cells, these CpG sites were exclusively located within the enhancer regions. whereas all six sites close to the TATA box and the cap site were consistently methylated. For all individual proviruses, the following rules applied. The level of methylation at the enhancer sequences was lower in the respective tissues of adults compared with tissues of fetuses, and methylation was lower in brain than in kidney and lower at the proviral 3′ than 5′ end. The chromosomal position of a given provirus strongly affected the extent of developmental demethylation (see below).

Enhancer sequences have been identified in viral and cellular genes (for reviews, see references 30, 39, and 41). At present, the mechanism by which these sequences exert their function is not understood; however, some of their known characteristics such as specific binding to cellular proteins (40, 44), acquisition of altered chromosome conformation (29, 45, 56, 57) and possibly altered DNA configurations (37) might account for the enhancer sequence specific demethylation events. Indeed, specific sites that were found previously unmethylated in genes coding for rRNA spacer sequences of *Xenopus* sp. (33) and in a rabbit beta-globin gene (13) have been recently identified as protein binding sites (11, 40).

The inherited stability of methylation patterns is most likely accomplished by the action of a maintenance methylase (49, 58). Loss of methyl groups probably occurs as a postreplicative event and may be caused by sterical interference of cellular components with the maintenance methylase. Our observations therefore indicate that enhancer sequences, but not other sequences in the M-MuLV genome, may have an intrinsic ability to perturb the interaction of the methylase with DNA.

De novo methylation of LTR sequences in early embryonic cells. All CpG dinucleotides of all M-MuLV genomes that were partially or completely unmethylated in sperm became completely de novo methylated at day 12 of gestation, whereas later in development, changes of methylation pat-

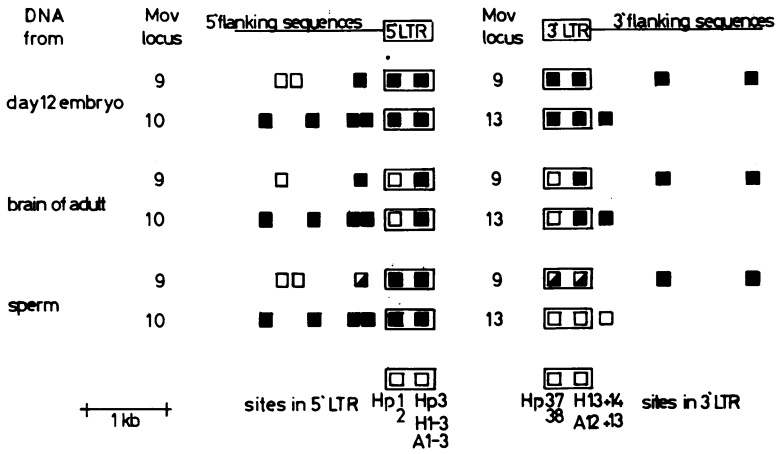

FIG. 7. Methylation of M-MuLV LTRs and of flanking cellular sequences. The methylation of *Hha*I (H), *Ava*I (A), and *Hpa*II (Hp) sites in M-MuLV LTRs (open rectangle) present in Mov mice (Fig. 3) is presented in conjunction with the methylation of individual sites in adjacent cellular sequences that were analyzed similarly with probes derived from Mov loci (Fig. 2B and 6A and B). The positions (see scale) of individual sites relative to the LTRs are marked by quadrants, which are filled, half filled, or left uncolored with methylated to 80% or more, between 20 and 80%, or less than 20%, respectively. The partial methylation of the 5′ site in Mov-9 sperm was detected by *Kpn*I-*Hha*I digestion with the HpX probe (data not shown). The analysis of the 3′ site in Mov-13 DNAs has been presented elsewhere (24a).

terns occurred by demethylation events (Fig. 3). This observation and the de novo methylation of enhancer sequences in the multiple M-MuLV genomes present in infected EC cells argue that enhancers become modified independently of the chromosomal position of a provirus. Our data furthermore suggest that de novo methylation in early pluripotent cells is not restricted to repetitive sequences (6), to DNA injected into mouse oocytes, or to retroviral genomes integrated into 4- to 16-cell mouse embryos (for a review, see reference 24), but also occurs in germ line-transmitted single-copy sequences. Furthermore, we recently found that unique cellular sequences adjacent to M-MuLV integration sites become de novo methylated during early mouse development (24a).

In normal mouse DNA, an almost constant fraction of 20 to 30% CpG dinucleotides are unmethylated in preimplantation embryos, EC cells, and tissues of the adult (48), which indicates that de novo methylation may occur at only a limited number of sites. Thus far, de novo methylation of unique cellular sequences in early mouse development has not been reported, nor is the exact time course of this modification at M-MuLV enhancer sequences known. EC cells, which resemble cells of the preimplantation embryo (36), have the capacity to methylate at these sequences, suggesting that de novo methylation in the Mov mice might occur before day 12 of embryogenesis. M-MuLV enhancer sequences, which do not function in EC cells (34), may not be able to bind specific cellular proteins in the early mouse embryo and may not, in contrast to most cellular sequences, be protected against de novo methylation. If a de novo methylase is part of the normal genetic program in early development, its function may be to correct demethylation events in the germ line which were introduced by errors of the maintenance methylase, to preserve the methylation level of a species (21).

Demethylation is dependent on the chromosomal position of the provirus. The extent of developmental demethylation of LTR sequences was strongly influenced by the chromosomal integration site of a particular proviral genome (Fig. 3). This position-dependent demethylation, however, was not accompanied by a similar change within the flanking cellular sequences, nor did there exist an obvious correlation between both methylation patterns (Fig. 7). Thus we did not obtain evidence for a "spreading" of demethylation from cellular DNA into retroviral genomes, as has been proposed elsewhere for position-dependent differences of methylation in endogenous mouse mammary tumor virus genomes (14). Instead, our data indicate that enhancer sequences are their own determinants for demethylation, as has been found for spacer sequences of *Xenopus* genes coding for rRNA, or for the developmentally regulated demethylation in the chicken globin locus, which is restricted to sequences within the gene (56).

The position-dependent lack of demethylation of some murine sequences coding for rRNA (2) and of a translocated human globin gene (31) was correlated to the presence of the sequences in an inactive chromosomal domain. In Mov-13 mice, however, which carry the provirus in the alphal(I) collagen gene (43), developmental changes of chromatin conformation concomitant with the demethylation of the enhancer sequences were seen in neither the viral or the flanking cellular sequences (4). Thus, position-dependent demethylation of M-MuLV enhancer sequences may be related to structural characteristics of chromatin conformation which cannot be recognized by present methods of analysis.

The preferential demethylation at the 3′ over the 5′ enhancer region in six different proviruses cannot be explained by differences in methylation patterns in flanking sequences or by differences in chromatin configuration (4). This pattern may reflect an intrinsic property of the retroviral genome, for example, the slightly different distance from the 5′ and 3′ enhancer sequences to the viral cellular junction.

Position-dependent methylation and expression of M-MuLV genomes. Ample evidence indicates that DNA methylation affects the expression of endogenous M-MuLV genomes. While M-MuLV genomes that were introduced experimentally into day-8 embryos replicated efficiently, remained unmethylated, and were infectious (26), the genetically transmitted M-MuLV genomes were not expressed before day 16 of development (22), were completely methylated at the 5′ LTR and at the coding regions early in development

(Fig. 3), and were not infectious (7, 18, 52). In addition, activation of repressed proviral genomes could be induced by treatment of mice with 5-azacytidine, a drug that reduces the level of methylation in cellular DNA (22a). Therefore the demethylation of proviral sequences, including the regulatory regions in the 5′ LTR, seems to be a prerequisite for provirus activation. The transcriptional inactivity of the replication-defective M-MuLV proviral genomes of Mov-10 (22a) and Mov-5 (T. Berleth, personal communication) mice, genomes which are virtually unmethylated at their 5′ enhancer regions (Fig. 3), indicates that demethylation of these sequences is not sufficient for gene expression. However, the position-dependent demethylation of these sequences may be a necessary event which enables the enhancer to respond to regulatory signals. Such signals may lead to virus activation in specific cell types and may become detectable only in mouse strains which carry nondefective M-MuLV proviruses and therefore can develop viremia. The results of this study are in agreement with this hypothesis. In adult mice, a low level of methylation at the 5′ enhancer region of the Mov-13 provirus that is activated in a subpopulation of cells of day-16 embryos was in contrast to a high level of methylation in the Mov-1 provirus that becomes activated only after birth (Fig. 3; 22). Demethylation of enhancer sequences occurs progressively during development and may be initiated in a few embryonic cells. Such demethylation events would escape the detection by restriction enzyme analysis, which showed a high level of methylation at the 5′ LTR in embryos of all Mov strains (Fig. 3).

Position-dependent expression of foreign genes that have been experimentally introduced into the germ line of mice has been observed frequently (5, 15, 22, 32, 38, 51) but is not understood molecularly. The modulation of sequence-specific demethylation events at specific chromosomal positions and by tissue-specific influences similar to those described here for the genetically transmitted M-MuLV genomes might be one molecular parameter to explain these observations.

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